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We have demonstrated in previous studies that low-energy laser reduces injury-induced deficits caused by acute partial injury of the rat optic nerve. The results of in-depth analysis suggest that the laser effect in this model is of a neuroprotective nature. We have devoted the time lapsed since approval of funding of the present study to characterization of the optic nerve partial lesion model, in an attempt to determine whether the progressive degeneration which occurs subsequently to the primary insult and continues in the absence of any external insult, is of a self-perpetuating nature, and whether such progressive degeneration follows any topographical pattern and whether any such pattern might be a reflection of the severity of the primary injury itself. By clarifying the above points, we may turn our model into an ideal model for studying the neuroprotection of the optic nerve and retinal ganglion cells from injurious conditions.

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Michael Schuh

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INTRODUCTION

Central nervous system (CNS) injury inevitably leads to a more widespread loss of function than would be expected to result directly from the cause of the injury itself. This excessive loss, whereby the primary loss of function spreads to neurons that were spared the direct effects of the injury, is known as "secondary degeneration". The occurrence of secondary degeneration has only recently been discovered following brain lesion, having been studied to a lesser extent following white matter lesions in the CNS, i.e. axonal injury.

There is a consensus that curtailing the spread of damage might have strong therapeutic implications both for acute and chronic injuries. Despite the efforts invested in this field and promising pre-clinical studies, no clinically effective therapy has been found. It is now conjectured that the lack of success to date is partially due to the model used in the pre-clinical studies which was either not optimally defined or whose results were not clearly understood in terms of the quantitative dissociation between the primary and secondary insults inflicted.

We have previously demonstrated that low energy laser has a beneficial effect on damage to the optic nerve. We propose, within the framework of our studies and with US Army support, to fully characterize the optic nerve model as a working model for neuroprotection, in order to understand the functioning of low level laser as a potential neuroprotective treatment modality and in order to compare it to other potential neuroprotective treatments. In addition, we have suggested that when finding out modes of secondary degeneration of retinal ganglion cells and potential drugs that can halt the process we will try to examine whether the same drug would provide broader protection in cases of other retinal injuries such as laser-induced injuries. In ophthalmology, laser is the standard therapy for many sight impairing retinal disorders. In addition, laser is widely used and thus laser-induced injuries occur quite often.

This report summarizes the results obtained by us to date, with respect to the characterization of the optic nerve as a model for axonal neuroprotection. The report also describes some preliminary results suggesting that the compounds found to be protective for the optic nerve and retina using the partial optic nerve lesion are found to be effective in a model of laser-induced injury in the retina.

EXPERIMENTAL METHODS, ASSUMPTIONS AND PROCEDURES.

Animals

Animal utilization was in accordance with the ARVO resolution on the use of animals in research. Adult male Sprague-Dawley (SPD) rats weighing 300-400 g from the Weizmann Institute of Science animal house were anesthetized with Vetalar (ketamine, 50 mg/kg) and Rompun (xylazine, 0.5 mg/kg) administered intraperitoneally. Prior to tissue excision animals were sacrificed by an overdose of sodium pentobarbitone (170 mg/kg, intraperitoneally).

Stereotactic application of Fluoro-Gold

Two weeks prior to crush injury, rats were deeply anesthetized and placed in a small stereotactic instrument. The skull was exposed and two holes were drilled above the left and right hemispheres (6 mm posterior to the bregma and 1.2 mm lateral to the midline above the superior colliculus, SC) leaving the dura intact. Using a Hamilton injector, we injected Fluoro-Gold solution (5% in distilled water) in three depth points in the (SC) 3.8mm, 4 mm and 4.2 mm under the bony surface (2 μl/2 min for each point). After completion of the injection the wound was sutured. Two weeks later the animal's right eye was subjected to crush-injury.

Crush injury

With the aid of a binocular operating microscope, lateral canthotomy was performed in the right eyes of anesthetized rats. The conjunctiva was incised laterally to the cornea, the retractor bulbi muscle was separated and the optic nerve exposed. Using calibrated cross-action forceps, a moderate or mild crush injury (1-4) was inflicted on the nerve 2 mm from the eyeball, taking special care not to interfere with the retinal blood supply.

Morphological analysis

Immediately after injury or 2 weeks or 1 month later, a retrograde labeling procedure involving local application of the dye 4-(4-(didecylamino)styryl)-n-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes, Europe BV) distally to the site of the lesion was used to label the cell bodies of neurons that had escaped the lesion. Because of the specific site of dye application, this approach enables the user to distinguish between undamaged neurons and injured

neurons with still-viable retinal ganglion cells, as only neurons whose fibers are morphologically intact can take up dye that was applied distally to the lesioned site and transport it to their cell bodies. Counting the number of labeled ganglion cells thus gives a direct measure of the number of spared neurons. Labeling and measurement were carried out as follows: the right optic nerve was again exposed as for the primary insult, again without damaging the retinal blood supply. Solid crystals (0.2-0.4 mm diameter) of the fluorescent lipophilic dye 4-Di-10-Asp (5) were deposited 1-2 mm from the distal border of the injury site. Noninjured optic nerves were similarly labeled at approximately the same distance from the globe. One week after dye application the animal was given a lethal dose of pentobarbitone (170 mg/kg). The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labeled ganglion cells by fluorescence microscopy. Retinal ganglion cells were counted as described in Results. It should be noted that the efficiency of labeling by this procedure is less than 100%, meaning that the method does not yield an absolute number of intact neurons; it does, however, allow a reliable quantitative comparison between treated and untreated nerves. Normalizing the number of fibers relative to that of control noninjured nerves provides a quantitative indicator of the number of intact fibers after injury with and without treatment.

RESULTS

Direct quantitative proof of secondary neuronal death

Rats were subjected unilaterally to an acute partial optic nerve injury of varying severity. The numbers of directly injured neurons and of neurons that escaped direct injury were determined by retrograde labeling with 4-Di-10-Asp. Application of the dye distally to the site of the lesion restricts the labeling to neurons that are still intact. This labeling strategy ensures that the dye is not taken up by neurons that are injured when the crush injury is inflicted or by subsequently damaged neurons adjacent to the site of the crush injury or at any site between the crush injury and the cell bodies. Comparison of the numbers of apparently intact optic neurons in injured animals and in noninjured controls immediately after the injury, showed that neurons which retained their axonal integrity after injury degenerated gradually and progressively in the absence of any further external injury (Fig. 1). Moreover, comparison of the results obtained after moderate, mild or very

mild injury revealed that the less severe the primary insult, the smaller the number of neurons that subsequently underwent secondary degeneration and the larger the number of neurons that finally remained undamaged (Fig. 1). However, application of the dye distally to the primary site of the lesion involves infliction of a second lesion in order to introduce the dye.

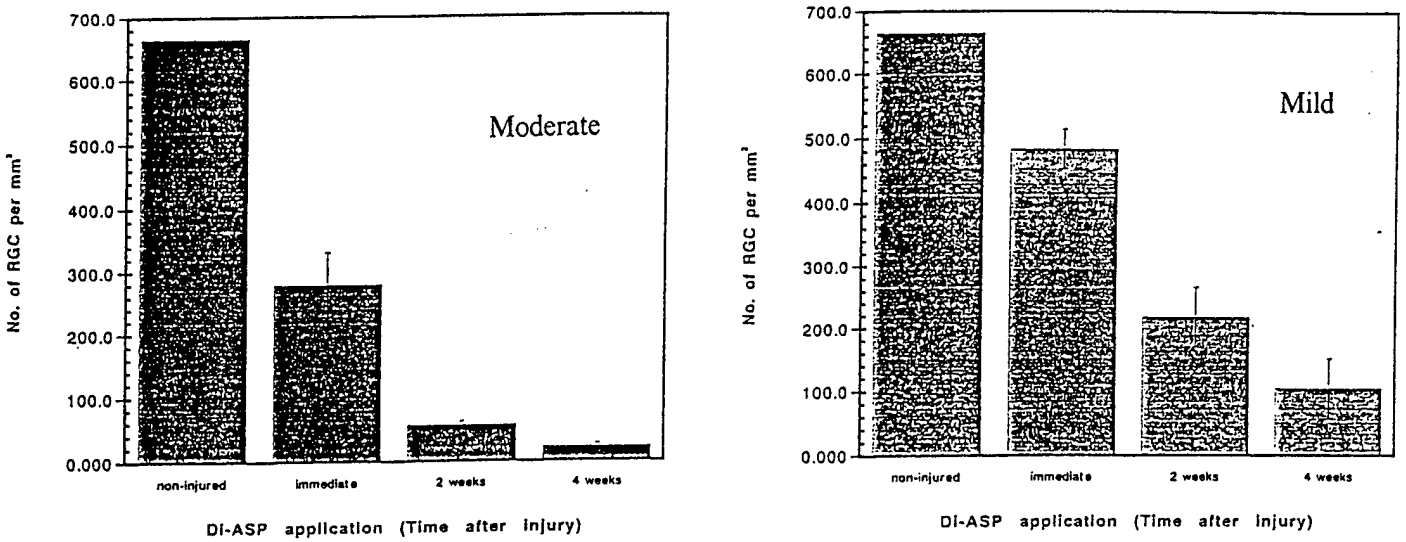

Figure 1. *Progressive loss of neurons left intact after axonal injuries of varying severity.* 4-Di-10-Asp was applied to optic nerves at different times after the nerves were subjected to crush injuries of varying severity. The retinas were excised 5 days later and flat-mounted. Labeled retinal ganglion cells (RGCs) from five fields in each retina (at approximately the same distance from the optic disk) were counted and their average number per mm^2 calculated. Bars represent means ±SEM of retinas of noninjured nerves(n=2); moderately injured nerves that were retrogradely labeled immediately (n=2), 2 weeks (n=6), and 4 weeks (n=8) after injury, and mildly injured nerves retrogradely labeled immediately (n=3); 2 weeks (n=3), and 4 weeks (n=4) after injury.

To ensure that this is a valid method of quantification and that the second axotomy required for the dye application does not itself cause further neuronal loss, we carried out a pre-labeling experiment in which all retinal ganglion cells were labeled with Fluoro-Gold prior to infliction of the crush injury. The animals were then subjected to a moderate crush injury, and in some of them this was followed 2 weeks later by an axotomy similar to that performed for dye application. Retinas were excised from all animals (both with and without the second axotomy) 3 or 7 days later and their retinal ganglion cell numbers were determined. As shown in Table 1, the

second lesion did not cause any further neuronal death during the 3 and 7 day time period, and there were no differences in the numbers of surviving retinal ganglion cells at 3 and 7 days. This experiment thus confirms the validity of morphometric analysis involving application of a dye distal to the site of lesion for quantification purposes.

Table 1: Surviving retinal ganglion cells following crush injury and with or without second axotomy

Days Post-Axotomy	Not axotomized (RGCs/mm^2)	Axotomized (RGCs/mm^2)
3	1015.5±60.5 (n=2)	974.0±10.54 (n=3)
7	918.5±66.5 (n=2)	971.67±38.8 (n=3)

Fluoro-Gold was injected stereotactically into the superior colliculus of naive animals. Two weeks later the nerves were exposed unilaterally and moderately crushed. After a further two weeks, some of the animals were re-anesthetized and the same optic nerves were re-exposed and subjected to a second lesion in the form of a transection distal to the primary insult. Retinas were excised from all animal 3 or 7 days later. Results are expressed as the mean number of retinal ganglion cells per mm^2 ±SEM. One-way analysis of variance revealed no differences between animals subjected to a second lesion and those that were not. (P=0.57).

Differential susceptibilities of neurons to primary insult and to secondary degeneration

In injured optic neurons, the rate of axonal degeneration was shown to be higher in neurons whose cell bodies are located at the retinal periphery than in those with cell bodies at the center (6). The difference in rate was attributed to differences in cell body vulnerability. To find out whether such differences also apply in the case of partial lesion, we mapped the retinal ganglion cell distribution in retina of noninjured nerve and in retinas of injured nerves 2 and 4 weeks after injury. The corresponding distribution patterns in injured optic nerves are shown in Figures 2 and 3.

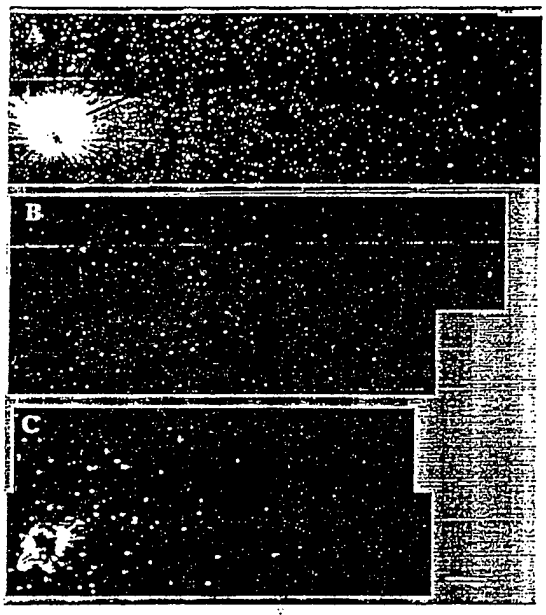

Figure 2. *Comparison of retrogradely labeled retinas of noninjured and injured nerves.* The figure shows representative micrographs of normal retina (A), retina of injured optic nerve labeled 2 weeks after the injury (B), and retina of injured optic nerve labeled 4 weeks after the injury (C). Bar = 300 mm.

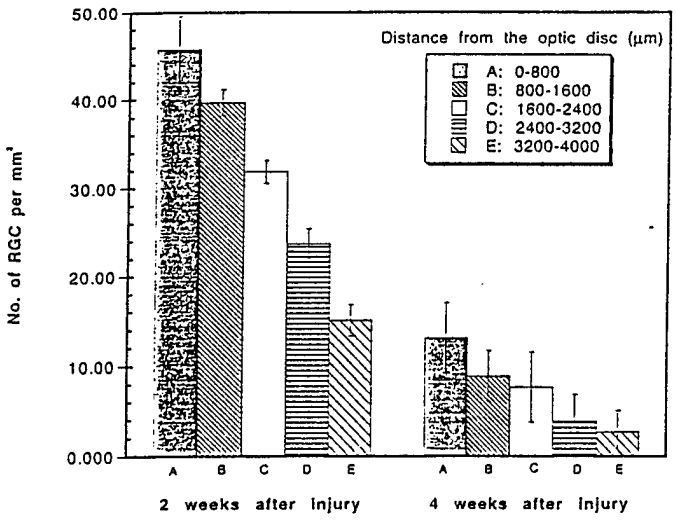

Figure 3. *Comparison of ganglion cell distribution in retinas of injured optic nerves 2 and 4 weeks after moderate injury.* Retinas were divided into five circular zones on the basis of their distance from the center (optic disk). The average number of RGCs per zone was calculated by counting them in four different fields in each zone. The graph presents means ± SEM of four retinas examined 2 weeks or 4 weeks after the optic nerve was injured.

Whereas in retinas of noninjured nerves the ganglion cells are distributed almost evenly in all retinal regions, in the retinas of injured nerves the ganglion cell loss is more pronounced at the periphery than close to the optic disk (Fig. 4).

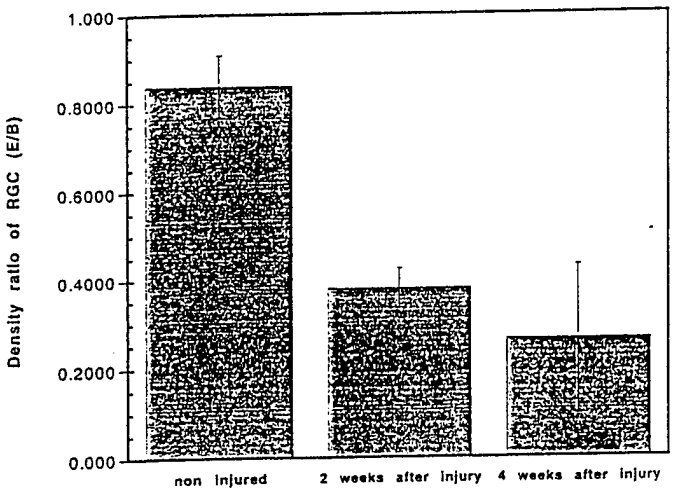

Figure 4. *Density of ganglion cells in the periphery relative to the center of the retina.* The distribution of RGCs in normal retina and in retina of injured nerves 2 and 4 weeks after moderate optic nerve injury was determined by comparing the ratios of cell densities in zone B and zone E in each retina. The zones correspond to those shown in Figure 4. The graph presents means ± SEM of the ratios obtained from two retinas of noninjured optic nerves and four retinas of optic nerves analyzed 2 and 4 weeks after injury.

These findings raised the question of whether topographical selectivity is triggered by the primary insult itself or by subsequent events. To address this question, we analyzed retinal ganglion cell topography immediately after injury. The results showed that immediately after a primary insult, even of mild severity, there is already a preferential loss of neurons whose ganglion cells are located in the retinal periphery (Fig. 5).

It therefore seems that the topographical selectivity is a reflection of the axons' susceptibility to the injury, the most vulnerable axons being those whose cells bodies are located peripherally. All subsequent degeneration is dictated by the topographical distribution of the acutely injured neurons.

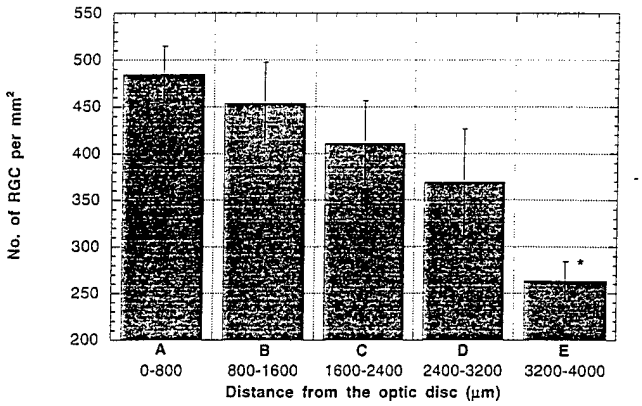

Figure 5. *Mechanical insult causes preferential loss of neurons whose ganglion cells are at the retinal periphery.* Retinas were retrogradely labeled immediately following mild injury and were whole-mounted 1 week later. Retinas were divided into five circular zones on the basis of their distance from the center (optic disk). The average number of RGCs per zone was calculated by counting them in four different fields per zone. The graph presents means ± SEM of four retinas. [ANOVA revealed a significant effect of the distance from the optic disk on the number of RGCs ($F=4.28$, $p= 0.028$). * Significant difference ($p< 0.05$) vs. the most central zone (zone A).]

Preliminary results of neuroprotection following laser-induced retinal injury

Argon laser lesions were created in the retinas of 86 DA rats and were followed immediately by intraperitoneal injections of MK-801 (2 mg/kg) or saline. The animals were killed after 3, 20 or 50 days and the retinal lesions were evaluated histologically and morphometrically. Photoreceptor-cell loss was significantly less in MK-801-treated rats than in control animals. Glutamate-receptor blockers should be further investigated as potential adjuvant therapy in retinal photocoagulation treatments.

DISCUSSION

Secondary degeneration following acute or chronic lesions or disorders of the CNS (7-12) is now widely recognized as an integral part of the response to any acute CNS event, such as trauma or ischemia. Intensive research efforts have therefore been directed towards uncovering the mediators of secondary degeneration and seeking ways to neutralize them, or their effects, or the

receptors with which they interact (13-18). These studies have opened the way to a new approach to the treatment of acute and possibly also of chronic CNS disorders, namely, neuroprotective therapy. Expectations engendered by the promising experimental results have not, however, been borne out by the outcome of clinical trials (19). A major obstacle to progress in neuroprotection can be attributed to the unsatisfactory empirical definition of secondary degeneration, on which much of the research has been based. Most of the studies of "secondary degeneration" do not provide direct quantitative proof of its occurrence, which would require separate quantification of the neuronal losses resulting from the primary injury and from secondary events. This issue becomes more critical in the case of white matter lesions such as axotomies and chronic neuropathies.

We show here that a partial lesion of the optic nerve leads to measurable secondary degeneration, which can be viewed as a distinct entity triggered by injury-induced mediators and not by the injury itself. Another characteristic of the degeneration observed in our study was its gradual, stepwise progression. This implies that neuroprotective therapy would need to be properly synchronized with the relevant physiological events, as protection given at time zero may not be sufficient for protection against delayed onset of degeneration. It should be noted that, in principle, secondary degeneration might start either in the axons of spared neurons or in the cell bodies. The process might occur in the following way: following the primary insult, injured neurons release into the environment toxic substances that spread to neighboring neurons. As a consequence these neurons, despite having escaped primary injury, undergo secondary degeneration, thereby releasing additional toxic substances into their environment and injuring more neurons (tertiary damage). The latter neurons release still more toxic substances, damaging yet other neurons, until eventually the process runs out of steam and reaches a "steady state". It seems, however, that under our experimental conditions even a mild insult caused a relatively massive primary loss of neurons and extensive secondary degeneration, apparently excluding the possibility of achieving an early steady state.

The results of this study may provide a plausible explanation for the progressive loss of neurons in chronic neuropathies even after the primary cause of the neuronal loss has been alleviated. Thus, for example, continuing loss of visual field has been observed in patients with glaucomatous neuropathy even after the intraocular pressure has been restored to normal (20,21). The fact that in some patients the disease is arrested whereas in others it continues to progress despite the normalization of intraocular pressure might be a reflection of the severity of the damage existing at the time of initiation of anti-hypertensive treatment (20). The topographical pattern of degeneration observed in the present study, i.e., loss of retinal ganglion cells at the periphery rather than at the center of the retina, is also reminiscent of that seen in glaucomatous neuropathy (22), where topographical differentiation and death of neurons have been attributed to size differences. Cell size may be one of the criteria for preferential loss of peripheral ganglion cells in mature retina, on the assumption that the largest retinal ganglion cells are the most susceptible (23).

It should be noted that although the crush injury inflicted in this study does not simulate glaucomatous or other human optic neuropathies from the point of view of etiology, it may resemble them in so far as the progression of nerve degeneration is not dependent on the nature of the primary cause. This further validates the use of a crush injury model for the study of secondary degeneration of neurons in chronic diseases such as glaucoma, where progressive deterioration continues after the primary cause has been eliminated. Optic neuropathies involving raised intraocular pressure, which can be diagnosed and treated by pressure reduction, may thus benefit from neuroprotective therapy.

The present study demonstrates the existence of secondary degeneration and strongly suggests that there is room for the development of neuroprotective therapy following acute or chronic injury where the primary cause can be identified and neutralized. Moreover, as secondary neuronal death is not only a self-perpetuating process but also a self-limiting one, the extent of nerve damage inflicted by acute trauma, or existing in chronic cases at the time of alleviation of the

primary cause, will determine the extent of the secondary loss. It is important to emphasize that neuroprotection should be considered as the protection of intact neurons immersed in a degenerative environment rather than as the protection of cell bodies of injured axons; the latter, even if successful, does not lead to recovery of function, which is the criterion of effective neuroprotection. In selecting a strategy for neuroprotective therapy, it should be borne in mind that the neurons which apparently escaped the primary insult may be marginally injured and hence more susceptible to the hostile environment created by degenerating neurons. Susceptibility might imply that the neurons possess "molecular memory" for injury in a way that changes the levels or localization of intracellular elements associated with survival or anti-death machinery. If this is the case, the use of neuroprotective compounds that only neutralize mediators of secondary degeneration might not be enough to rescue such neurons; they might need, in addition, an intracellular signal that would eliminate the "molecular memory" of the injury.

The fact that our preliminary results suggest that neuroprotective therapy is plausible in halting retinal degeneration following laser-induced injury is very encouraging, as it emphasizes that neuroprotection is a valid approach following various types of injury.

CONCLUSIONS

1. Partial lesion of the optic nerve leads to a self-perpetuating process of secondary degeneration.

2. The extent of damage is a function of the severity of the primary insult.

3. The loss of retinal ganglion cells occurs in a specific topographical pattern, with the periphery taking precedence over the center.

4. The optic nerve partial injury model is suitable for further characterization of the mechanism underlying the low-energy laser effect, for scanning of other potential neuroprotective compounds, for making comparative studies, for detection of the therapeutic window and for uncovering the molecular mechanism at work.

5. Laser-induced injuries to the retina, like any other injury, initiate secondary degeneration that operates in a self-perpetuating manner and perhaps involves similar mediators, e.g. excitatory amino acids as in the case of a trauma starting at the optic nerve head.

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